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CD49f$^+$ mammary epithelial cells decrease in milk from dairy cows stressed by overstocking during the dry period

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The work reported in this Research Communication describes the modification in epithelial cell populations during the first and the last month of milking in Holstein Friesian cows that have undergone different management during the dry period, and we report the differential expression of CD49f$^+$ and cytokeratin18$^+$ cell subpopulations. Twenty six cows were randomly divided into 2 balanced groups that were housed at stocking density of either 11 m^2 (CTR) or 5 m^2 from 21 ± 3 d before the expected calving until calving. Cells collected from milk samples taken in early lactation and late lactation were directly analysed for CD45, CD49f, cytokeratin 14, cytokeratin 18 and cell viability. We observed a differential expression with a significant reduction in CD49f$^+$ ($P < 0{\cdot}01$) and cytokeratin 18$^+$ ($P < 0{\cdot}05$) cells in early lactation. Differences were still evident in late lactation but were not significant. These observations suggest that mammary epithelial cell immunophenotypes could be associated with different animal management in the dry period and we hypothesise they may have a role as biomarkers for mammary gland function in dairy cows.

Keywords: integrin alpha 6, stress, biomarker, dairy cow

Increased stocking density is a common practice among dairy producers. One of the main tools to evaluate the effect of such management is to determine whether a stressful threshold is exceeded, triggering a number of changes such as activation of the sympathetic nervous system and hypothalamic-pituitary-adrenal axis, both of which are well-known sources of biomarkers for animal welfare (Prunier et al. 2013). Very recently it has been demonstrated that overstocking during the dry period in Holstein Friesian dairy cow is also associated with changes in DHEA (Fustini et al. 2017). However, the determination of hormonal patterns to evaluate stressful situations presents some difficulties for the sampling and comparison of hormone levels in a given time interval. Also, it would be interesting to include the ability to insert other physiological parameters that are to some extent related to the animal's wellbeing. We have reported the expression of epithelial cell precursors and fully differentiated cells in bovine milk, highlighting possible variations in the number and features of mammary epithelial cell (**MEC**) subsets in dairy cows (Baratta et al. 2015). MEC are found in milk, caused by shedding during the lactation phase, but the range of cell frequency differs from total somatic cell count (**SCC**) if only the live cell fraction is analysed. The total amount of somatic cells in milk is affected by different factors, such as species, breed, lactation phase, milk yield, individual animal differences and management practices (Rupp et al. 2000). A specific pattern of epithelial cell types has been found in the milk according to the stage of lactation. Cell types include an inner layer of cytokeratin 18 (**K18**)$^+$ luminal cells and an outer layer of cytokeratin 14 (**K14**)$^+$ myoepithelial cells while CD49f$^+$ cells are probably derived from a more primitive stage of cell differentiation (Martignani et al. 2015). In this study we show different mammary epithelial cell types present in milk of dairy cows that have undergone overstocking during the dry period and hypothesise that specific cell type variations may be related to stressful management.

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Materials and methods

Animals, housing and diet

Twenty six Holstein dairy cows were enroled in thisexperiment. All animals were housed at the farm of the University of Bologna (Ozzano Emilia, Italy) and used according to EEC animal care guidelines. The experimental procedures had been approved by the Ethical Committee of Bologna University. Animals were randomly divided into two groups (13 animals each), balanced for number of lactations, BCS (body condition score) and expected date of calving. Cows in the far-off phase of the dry period (from 60 to 21 d before the expected calving date) were housed together in a bedded-pack and received water and grass hay ad libitum. From 21 ± 3 d until calving animals were housed in two bedded-pack groups where they had ad libitum access to water and were fed daily using total mixed ration. After calving cows were housed together in a bedded pack area for the first 2 weeks of lactation and then moved to a free-stall pen for the rest of lactation. The total mixed rations (TMR) were fed approximately at 7 am for lactating cows and 9 am for dry cows.

Experimental design, blood sampling and hormone assays

Animals, dried off 8 weeks before the expected calving, were housed in pens with the same size (22·5 m^2 in total with 13·5 m^2 of resting area and 9 m^2 of feeding area) but in different crowding conditions due to the introduction in the pen of heifers (interference animals) having a body weight of 450–550 Kg. Control condition (CTR) had 2 animals per pen (one animal of the study with an interference animal) with 11 m^2 each, while the overstocked condition (OS) had three interference animals in the same pen with 5 m^2 for each animal. Cows were allocated to CTR or OS groups based on parity, at 21.d before expected calving dates. The resting area is a deep-bedded pack with straw added twice a day. On days −30, −21, −7 before and 4, 10, 30, 60 relative to calving blood samples were collected from each cow for the determination of plasma DHEA and cortisol (C) concentrations by RIA.

Flow cytometry analysis: sample processing

Quarter foremilk samples were obtained in accordance with the Veterinary Services Standards of the Italian National Health Service, branch of the Ministry of Health. Before morning milking, teats were scrubbed with 70% ethanol and the first 2 strips of milk were discarded. Aliquots of 200 ml of milk per udder were collected aseptically. Cells were collected and analysed according to previously reported methods (Baratta et al. 2015). Briefly, the determination of epithelial subpopulations in milk was carried out utilising a 6-color flow cytometry assay. Anti-CD45 antibody (VMRD Inc., Pullman, WA) was used to gate immune cells, anti-human-CD49f–FITC antibody (anti-h-α-integrin-6-FITC, Novus Biological, Littleton, CO), monoclonal anti-CK peptide 18 antibody (clone KS-B17·2, Sigma, St. Louis, MO), and anti-CK14 antibody (Covance, Life Technology, Thermo Fisher). Stained samples were analysed using an Attune Acoustic Focusing Cytometer (Life Technologies). Cells without antibody labelling served as a negative control and were regarded to be a measure for background fluorescence. Fluorescence Minus One (FMO) controls were used to identify data spread due to the multiple fluorescent signals (2000; Bayer et al. 2007). Epithelial cells were identified and counted in the total living $CD45^-$ cell population.

Statistical analysis

The two groups of cows were compared on the following variables: living cells, $CD49f^+$, $K14^+$, $K18^+$, and $K14^+18^+$; values were collected at the beginning and in the last month of lactation. Considering that all variables were frequencies, non-parametric tests were performed for all the analyses. In particular, Mann-Whitney *U* test was chosen and, firstly, variables were compared between the groups of cows at the first month of lactation. Secondly, the same analyses were repeated for measures collected in the last month of lactation, in order to explore differences in significant results. Results were considered significant when associated at least to $P < 0{\cdot}05$ for all the comparisons.

Results

Hormone concentrations

In overstocking group (OS) DHEA significantly ($P < 0{\cdot}01$) increased compared to CTR group at day −7 (2·13 ± 0·63 *vs.* 1·47 ± 0·46 pmol/ml) while C did not differ between CTR and OS group (data reported in Supplementary Fig. S1)

Frequency of epithelial subpopulations during the first month of lactation

Figure 1a shows total living cells (ranging from 66 to 78%) detected in the somatic cell population in early lactation, identified as $CD45^-$ cells, in Holstein Friesian cows in response to stress induced by overstocking (OS) or not (CTR) during the dry period. A significant difference between the two groups was observed in $CD49f^+$ cells ($P < 0{\cdot}01$) with a decrease in OS group from 20 to 5%. Interestingly, we observed a significant difference ($P < 0{\cdot}05$) in the level of luminal cells ($K18^+$) with a decrease in OS group. Finally, no differences were detected in myoepithelial ($K14^+$) and $CK14^+/CK18^+$ cells.

Frequency of epithelial subpopulations during the last month of lactation

Figure 1b shows total living cells ranging from 63 to 75% detected in the somatic cell population in late lactation,

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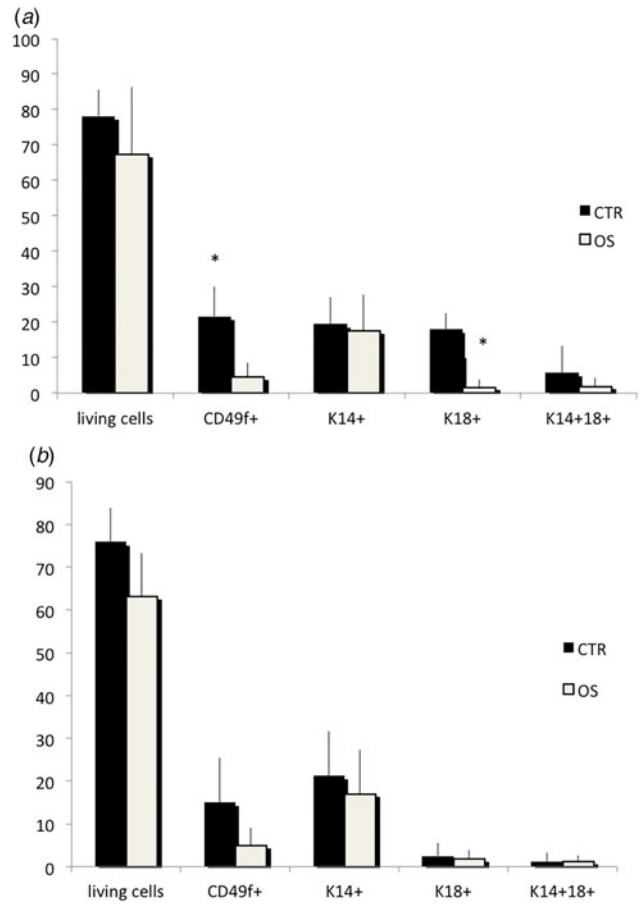

Fig. 1. Frequency in percentage of cell viability and of epithelial cell subpopulations in bovine milk during the first month of lactation (a) and during the last month of lactation (b) in control (CTR) and overstocked condition (OS) groups. Cell subpopulations are identified according to the positive expression of CD49f, K14 and K18. *$P < 0{\cdot}05$ or better. Error bars represent S.D.

identified as $CD45^{-}$ cells, in cows that were exposed to stress during the dry period induced by overstocking (OS). A tendency to a decrease in OS group was observed without reaching a statistical difference ($P = 0{\cdot}066$). Luminal cell ($K18^{+}$) were present at low frequency in both groups (2–3%) while myoepithelial cells ($K14^{+}$) still showed a greater concentration ranging from 18 to 21%. Finally, no differences were detected in myoepithelial, luminal and $CK14^{+}/CK18^{+}$ cells between the two groups.

Milk yield in response to treatment over transition period

Mean milk yield (kg/d) in response to treatment over the transition period was not different among treatments (Table 1). Among cows, treatment did not differ regarding previous lactation 305-d mature- equivalent milk yield (CTR = 10·1 ± 215·1 kg, OS = 9·5 ± 187·7 kg; $P > 0{\cdot}05$).

Table 1. Mean ECM yield (kg/d) in response to treatment experienced in the transition period

Week after calving	Control (CTR)	Overstock condition (OS)	S.E.M.	*P*-value
1	23·5	22·3	1·4	0·65
2	34·9	31·9	1·5	0·13
3	35·8	34·4	1·6	0·23
4	37·1	35·9	1·3	0·18

Discussion

It has recently been reported that DHEA secretion is affected in response to overstocking during the dry period in Holstein Friesian cows (Fustini et al. 2017). We reported that DHEA concentrations were affected only during the dry period, when the stressful stimulus was applied, while no differences in DHEA secretion were observed during the first two months of lactation. Since the placenta seems the most important DHEA source in the late pregnant cow (Gabai et al. 2004), it is possible that overstocking stimulates the release of DHEA from the maternal-foetal units through a still unknown mechanism. In the present work we cannot investigate the source of this metabolite, however, we can confirm that in dairy cows DHEA plasma levels are affected during the last part of pregnancy by stressful management like overstocking that often occurs during the dry period.

We mainly focused our attention on the frequency and differential expression of epithelial cells subpopulations in milk. We have previously reported the expression of epithelial precursors and fully differentiated cells according to the phase of lactation (Baratta et al. 2015). We report now further information that leads us to consider the hypothesis that different distributions of MEC subpopulations may provide more detailed information on the physiology of the mammary gland during lactation in dairy cows. In particular, our data suggest that stressful situations can affect the somatic cell subpopulations. We compared cows that received or did not receive the stressful experience of overstocking (monitored by change of DHEA) during the last days of dry period, making measurements during the first and the last month of the lactation period. We observed a different pattern of expression between the two groups of animals in the first month of lactation but not at the end of the physiological period indicating that the stressed cows showed a lower expression in $CD49f^{+}$ and $K18^{+}$ cell populations. We were interested in the CD49f population evaluation since they belong to more primitive MEC (mammary precursors). They appear to decrease during the decline of lactation and in this way may exert a role in the reduction of the mammary secretory function, which adjusts the number of active secretory cells. We hypothesise that this subpopulation may be considered the signal of a reduction in mammary efficiency. The presence of CD49f positive cells, even if in a low number, may be related also to the reduction in the myoepithelial compartment that indicated the modification of the myoepithelial genetic program

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(Garbe et al. 2012). CD49f is an integrin subunit α6 that regulates signalling pathways in a variety of cellular activities and it has been shown to be a component of a feedback circuit that regulates the myoepithelial phenotype in mammary epithelial cells from humans and mice (Deugnier et al. 1999; LaBarge et al. 2009) suggesting that the basal regulatory machinery may be disrupted in myoepithelial cells and inappropriately engaged in luminal epithelial cells, maybe during the aging process. We did not observe a significant difference in K14/K18 double positive cells, in term of activation of regenerative functional tissue of mammary gland, in particular during the final phase of lactation. On the contrary, we have observed a difference in $K18^{+}$ cells with a decrease in OS group during the first month of lactation. This subpopulation is specifically linked to the secreting cells since they are referred to as luminal cells. We would expect this difference to be associated with a reduction in milk yield during the period of milking, although we did not observe any decrease in milk production. The exposure to stressful conditions might influence the numerical relationship between luminal cells that produce milk in the mammary gland and epithelial cells that are shed in milk. One aspect that deserves to be thoroughly investigated is the number of functional cells found in milk needed to detect an effect on milk production.

Conclusions

In conclusion, we report the expression of epithelial precursors and fully differentiated cells during the first month of lactation in dairy cows that were overstocked during the previous dry period, highlighting variations in the number and features of MEC subsets in milk. Although we were not able to detect a correlation with milk production, it remains interesting to observe that overstocking associated with hormonal pattern during dry period shows different modulation of somatic cells during the lactation. Further studies are necessary to determine if different distributions of MEC subpopulations may provide more detailed information on the physiology of the mammary gland during lactation in dairy cows and, potentially, have an application to evaluate mammary gland functionality as biomarkers.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029917000589.

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